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DOMESTIC ANIMAL ENDOCRINOLOGY

Domestic Animal Endocrinology 27 (2004) 125-140

www.elsevier.com/locate/jnlabr/dae

The role of insulin, glucagon, dexamethasone, and leptin in the regulation of ketogenesis and glycogen storage in primary cultures of porcine hepatocytes prepared from 60 kg pigs[☆]

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Received 21 May 2003; accepted 13 February 2004

Abstract

A study was conducted to elucidate hormonal control of ketogenesis and glycogen deposition in primary cultures of porcine hepatocytes. Hepatocytes were isolated from pigs (54–68 kg) by collagenase perfusion and seeded into collagen-coated T-25 flasks. Monolayers were established in medium containing fetal bovine serum for 1 day and switched to a serum-free medium for the remainder of the culture period. Hepatocytes were maintained in DMEM/M199 containing 1% DMSO, dexamethasone (10^{-6} or 10^{-7} M), linoleic acid (3.4×10^{-5} M), and carnitine (10^{-3} M) for 3 days. On the first day of serum-free culture, insulin was added at 1 or 100 ng/ml and glucagon was added at 0, 1, or 100 ng/ml. Recombinant human leptin (200 ng/ml) was added during the final 24 h; medium and all cells were harvested on the third day. Concentrations of acetoacetate and β -hydroxybutyrate (ketone bodies) in media and glycogen deposition in the cellular compartment were determined. Ketogenesis was highly stimulated by glucagon (1 and 100 ng/ml) and inhibited by insulin. In contrast, glycogen deposition was stimulated by insulin and attenuated by glucagon; high insulin was also associated with a reduction in the ketone body ratio (acetoacetate: β -hydroxybutyrate). High levels of dexamethasone stimulated ketogenesis, but inhibited glycogen deposition at low insulin. Culture of cells with leptin for 24 h, over the range of insulin, glucagon, and dexamethasone concentrations

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^{*} Mention of trade name, proprietary product or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture or imply its approval to the exclusion of other products or vendors that also may be suitable. Presented in part at the ASAS Annual Meeting, Indianapolis, IN, 2001. Abstract # 1781.

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had no effect on either glycogen deposition or ketogenesis. These data suggest that while adult porcine hepatocytes are indeed sensitive to hormonal manipulation, leptin has no direct influence on hepatic energy metabolism in swine.

Published by Elsevier Inc.

Keywords: Pig hepatocytes; Ketogenesis; Ketone body ratio (KBR); Glycogen; Leptin

1. Introduction

Interest in hepatocytes prepared from pigs has increased markedly because of the potential of using these cells in liver rescue devices for humans. Although a great deal is known about xenobiotic metabolism and albumin synthesis in primary monolayer cultures of pig hepatocytes [1–3], few basic studies have been conducted to elucidate how hormones control basic hepatic functions and energy metabolism in these cultures. Previous metabolic studies focused on freshly prepared hepatocytes from newborn or young pigs [4–10] while studies conducted with cultured hepatocytes from growing pigs older than 3 or 4 months of age (50–70 kg) are lacking. The primary objectives of this investigation were to determine ketogenic and glycogenic capacity of monolayer cultures of porcine hepatocytes and their regulation by hormones that are integrally involved in the utilization and partitioning of dietary nutrients.

In rat hepatocytes, insulin has been shown to increase glycogen deposition [11–16]. However, the influence of insulin on ketogenesis in hepatocyte cultures has not been systematically investigated. While short-term glucagon addition has been shown to have no effect on ketogenesis in pig hepatocytes [4], its long-term roles on ketogenesis and glycogen deposition have not been previously determined. The addition of glucocorticoids (specifically, dexamethasone) has long been known to be important for maintenance of differentiated hepatocyte function in vitro [17], however, there is no available data on the role of dexamethasone in ketogenesis and glycogen metabolism in pig hepatocytes.

Leptin, a polypeptide hormone secreted primarily by adipose tissue, appears to play a major role in feed intake and energy metabolism. In normal rat hepatocytes it has been suggested that leptin acts in concert with insulin to enhance glycogen deposition by inhibiting glycogen degradation [16]. In contrast, leptin may also antagonize insulin effects on glucose and lipid metabolism. For example, leptin increases hepatic glucose production in wild type mice [18] and PEPCK mRNA in rats [19,20]. Early studies in genetically obese (ob/ob) mice showed a hepatic insensitivity to glucagon [21] which suggests that leptin may be necessary for glucagon-induced glycogenolysis. In contrast, Zhao et al. [22] showed that leptin suppresses glucagon-induced cAMP responses in rat hepatocytes, while the presence of glucagon appeared to completely block any influence of leptin on glycogen metabolism [16]. Leptin treatment has been shown to decrease and increase ketone production in lean and obese ob/ob mice, respectively [23], and circulating levels of leptin and ketones appear to be inversely related [24]. However, no studies have been conducted to directly assess the affects of leptin on ketone production by hepatocytes. Therefore, we developed a hepatocyte isolation and culture system utilizing pigs, that is highly sensitive to levels of insulin and glucagon, to assess the possible interactions of leptin on hepatic metabolism in the pig.

2. Materials and methods

2.1. Hepatocyte isolation

Five crossbred barrows (54–68 kg) were stunned by electric shock and exsanguinated. Livers were immediately excised and the left lateral lobe was removed. Care and treatment of all pigs were approved by the Institutional Animal Care and Use Committee of the US Department of Agriculture. Hepatocytes were isolated as previously described by Caperna et al. [25], except only a small portion (approximately 80 g) of the lobe was used. A cannula was inserted into a section of the portal vein and the cut edges of the lobe were loosely sewn with 00 silk to facilitate tissue perfusion. Collagenase-digestion buffer was modified to contain a 1:1 mixture of M199 and 100 mM HEPES-buffered saline containing 67 mM NaCl, 4.8 mM CaCl₂, and 6.7 mM KCl. The buffer mixture was amended with 0.1% BSA, 0.5% glucose, 0.075% collagenase (class 2, Worthington Biochemical Corp., Lakewood, NJ), and 5 μ g/ml bovine insulin. This enzyme solution was re-circulated through the liver piece for 80 min. Viability of isolated hepatocytes from animals in this study was 87.4 \pm 1.2% (trypan blue dye exclusion) and cell yield from each liver preparation was 1.4 \pm 0.2 \times 109 viable hepatocytes (n=5).

2.2. Cell culture

Hepatocytes (4×10^6) were seeded (day 0) into vented T-25 flasks pre-coated with 150 μg pig tail tendon collagen. Cells were initially maintained at 37 °C in 5% CO₂ in basal medium (1:1 mixture of low glucose DMEM (3.5 g/l NaHCO₃) and M199 (1.25 g/l NaHCO₃) amended with β -mercaptoethanol (10⁻⁴ M), glutamine (4 mM), and antibiotics (gentamicin, penicillin, streptomycin, amphotericin B) containing ITS (insulin: transferrin:selenium, 2.5 µg/ml: 2.5 µg/ml: 2.5 ng/ml) and 10% fetal bovine serum (FBS). Following a 3 h attachment period, flasks were washed twice with HEPES-buffered saline and basal medium containing 5% FBS and ITS was added to each flask. On the following day, flasks were washed twice with HEPES-buffered saline and medium was replaced with serum-free basal medium containing 10⁻⁶ M dexamethasone, 1% DMSO, albumin:linoleic acid (1 mg/ml: 9.4 µg/ml), 10^{-3} M carnitine, and 10^{-7} M Na₂SeO₃. All media contained 2 g/l glucose. Culture medium, glutamine, and antibiotics were obtained in liquid form from GIBCO-BRL (Rockville, MD) and all other reagents except where noted are from Sigma (St. Louis, MO). Peptide hormones (bovine insulin, porcine glucagon) were added as described below and recombinant human [rh]-leptin (Calbiochem, La Jolla, CA) was solubilized and stored at -80 °C until used. Duplicate flasks were used for each experiment and media was changed daily, following two washes with warm HEPES-buffered saline.

2.3. Analytical

The following hormone conditions were established on the first day of serum-free culture for all experiments: insulin (1 and 100 ng/ml); glucagon (0, 1, and 100 ng/ml); dexamethasone (10^{-6} M, except where noted). Recombinant human leptin (0 and 200 ng/ml) was

added for the final 24 h of culture. On the third day of culture, media was harvested following a 24 h incubation period and centrifuged prior to freezing. Cell monolayers were washed twice with glucose-free HEPES-buffered saline and cells from each flask were scraped off and homogenized by sonication in 2 ml of buffer (50 mM imidazole acetate, pH 7; 20 mM KF, 5 mM EDTA, 0.2 mM DTT). Media and cells were stored at -80 °C prior to analysis.

2.3.1. Ketone production

Ketone bodies in media were determined from samples collected on the third day of incubation following a 24 h period under the conditions outlined for each group. Because ketone production was expected to be low in media from pig hepatocytes, a sensitive micro-method in 96 well plate format was developed to analyze β -hydroxybutyrate (BHB) and acetoacetate (ACAC). Concentrations of ACAC and BHB were determined by a modification from Harano et al. [26] and Williamson and Mellanby [27], using a BHB-dehydrogenase-nicotinamide adenine dinucleotide (NADH)-coupled system. Lower limit of detection was 3 nmol of ACAC or BHB per milliliter of media following direct analysis in a fluorescence microtitre plate reader (HTS 7000, Perkin-Elmer, Norwalk, CT). Data are expressed as nmol of ACAC or BHB (or total ketones, ACAC + BHB) per milligram cell protein per 24 h.

2.3.2. Glycogen determination

Glycogen in cell homogenates was determined from samples collected on the third day of incubation following a 24 h period with the conditions outlined for each group by a modification of the method of Nguyen et al. [28]. Samples were incubated in the absence (background) or presence of amyloglucosidase (E.C. 3.2.1.3) for 1 h at 37 °C and pH 5.5, to liberate glucosyl units from glycogen. Glucose was subsequently assayed in microtitre plates by a modification of an enzymatic method (Sigma Diagnostics #18-UV). Background glucose content was subtracted to obtain cellular glycogen content and data were expressed as microgram glucosyl units released from glycogen per milligram cell protein.

2.3.3. Protein and DNA determination

Protein in cell homogenates was determined by a modified Lowry procedure following NaOH solubilization of TCA-precipitated material [29]. BSA was used as the protein standard. DNA was determined fluorometrically by a Hoechst dye binding assay [30]. Briefly, samples were extracted in $0.5 \, N \, NH_4 \, OH \, (in \, 0.1\% \, Triton \, X)$ for $10 \, min$ at $37 \, ^{\circ} \, C$, then diluted in $10 \, mM$ Tris, pH 7.0 amended with NaCl ($100 \, mM$) and EDTA ($10 \, mM$) prior to addition of dye (Hoechst 33258, Sigma). Samples were incubated at room temperature in the dark for $10 \, min$ and standards and samples were read with a fluorometer (AB2, Spectronic Unicam, Rochester, NY; excitation, $350 \, nm$; emission, $455 \, nm$).

2.4. Statistical analysis

Results are expressed as means \pm S.E.M. of duplicate flasks from the number of hepatocyte culture preparations indicated. Statistical analysis was performed using GLM ANOVA (5 \times 2 treatment array; insulin (1 and 100 ng/ml) and glucagon (0, 1, and 100 ng/ml) and rh-leptin (0 and 200 ng/ml)) and the means were separated by the Neuman–Keuls

multiple-comparison test (Number Cruncher Statistical Systems, Kaysville, UT). There was no significant influence of leptin addition to cultures for any parameters investigated and there were no treatment × leptin interactions. The indicated comparisons noted as percent changes between pre-planned comparisons have been determined by combining the control and leptin-containing cultures (1I, 0G versus 100I, 0G; 1I, 1G versus 100I, 1G; 1I, 0G versus 1I, 1G; 1I, 0G versus 1I, 10G; 1I, 1G versus 1I, 10OG and 100I, 0G versus 100I, 1G). An additional ANOVA was performed to determine the overall influence of insulin (high versus low) on total cellular protein and DNA in hepatocyte cultures.

3. Results

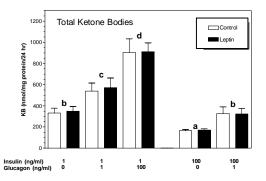
3.1. Ketones

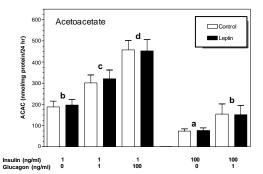
A pilot experiment was conducted to establish the overall rate and optimal conditions for ketone body (KB) production in cultured pig hepatocytes. Addition of insulin (1, 10, 100, 500 ng/ml) to hepatocyte cultures varying in glucagon concentration (1, 10, 100, 500 ng/ml) was associated with decreased ACAC and BHB production. In contrast, glucagon markedly increased ACAC and BHB production. Addition of either glucagon or insulin at 500 ng/ml had no additional influence on ketogenesis compared to 100 ng/ml, (data not shown).

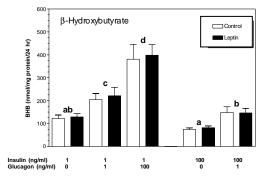
The specific influences of insulin, glucagon, and leptin on ketogenesis are shown in Fig. 1. Insulin had a significant inhibitory effect on KB production (nmol total KB/mg protein/24 h). When insulin was elevated from 1 to 100 ng/ml in the absence of glucagon (basal conditions), KB and ACAC production was decreased by 52 and 61%, respectively. A low glucagon concentration had no apparent influence on the inhibitory effect of insulin on ketogenesis since the levels of KB, ACAC, and BHB decreased by 43, 51, and 31%, respectively, as insulin was elevated from 1 to 100 ng/ml in media containing 1 ng/ml glucagon.

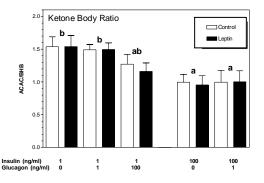
Addition of glucagon was associated with an overall increase in KB production. When hepatocytes were maintained in low insulin (1 ng/ml) without glucagon, addition of 1 and 100 ng/ml glucagon increased KB, ACAC, and BHB production by 65, 62, 69% and 165, 136, 210%, respectively. Compared to cultures containing 1 ng/ml insulin and 1 ng/ml glucagon, the addition of 100 ng/ml glucagon similarly elicited an increase in KB, ACAC, and BHB production (61, 46, and 83%, respectively). When there is no glucagon in the cultures and high insulin is present (100 ng/ml), addition of 1 ng/ml glucagon increased KB, ACAC, and BHB production by 95, 102, and 88%, respectively.

The redox potential of the mitochondria may be evaluated by the ACAC to BHB ratio (KBR). As BHB and ACAC freely penetrate the cell membrane, the KBR has been suggested to reflect specifically the proportion of free oxidized NAD⁺ to reduced NADH locally within the liver mitochondria [31]. In this experiment, KBR ranged between 0.96 and 1.54. Insulin significantly altered KBR. When hepatocytes maintained in the absence of glucagon and with 1 ng/ml insulin were compared to hepatocytes maintained with 100 ng/ml insulin, a decrease (1.54 versus 1.00) in KBR was observed. The presence of glucagon in the media (1 ng/ml) did not seem to influence the insulin effect (1.49 versus 1.00) on KBR. In contrast, in the presence of 1 ng/ml insulin, glucagon addition (1 or 100 ng/ml) did not significantly influence KBR. Addition of rh-leptin (200 ng/ml) to hepatocyte cultures for 24 h with dif-









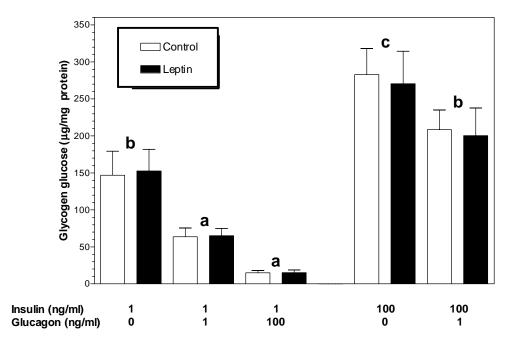


Fig. 2. Influence of insulin, glucagon, and leptin on glycogen deposition in porcine hepatocytes. Following indicated incubations and removal of media, cell monolayers were washed with glucose-free medium and cells were scraped and frozen. Glycogen was determined as outlined in Section 2. Data are means and S.E.M. for duplicate cultures from the five animals represented in Fig. 1. Means with different letters differ, P < 0.05.

ferent insulin (1 and $100\,\text{ng/ml}$) and glucagon (0, 1, and $100\,\text{ng/ml}$) concentrations did not affect KB production, nor did leptin impact upon KBR.

3.2. Glycogen

Glycogen content (µg glycogen glucose/mg protein) in hepatocytes increased by 85% when insulin was elevated from 1 to 100 ng/ml in the absence of glucagon (Fig. 2). The stimulating effect of insulin was greater (217%) when hepatocytes maintained in 1 ng/ml insulin and 1 ng/ml glucagon were compared to hepatocytes maintained in 100 ng/ml insulin and 1 ng/ml glucagon. In contrast, glycogen content was decreased by 57 and 90% when hepatocytes maintained with 1 ng/ml insulin and without glucagon were compared to hepatocytes with 1 ng/ml insulin and 1 or 100 ng/ml glucagon, respectively. When hepatocytes were cultured in 100 ng/ml insulin, the decrease in glycogen content was only 26%

Fig. 1. Influence of insulin, glucagon, and leptin on ketogenesis in porcine hepatocytes. Media was conditioned during the final 24 h of incubation and was harvested, prepared, and analyzed as described in Section 2. Glucagon and insulin were present at indicated concentrations for 72 h while rh-leptin was present for the final 24 h period. Data represent the means and S.E.M. for duplicate cultures from five animals. Means with different letters differ, P < 0.05.

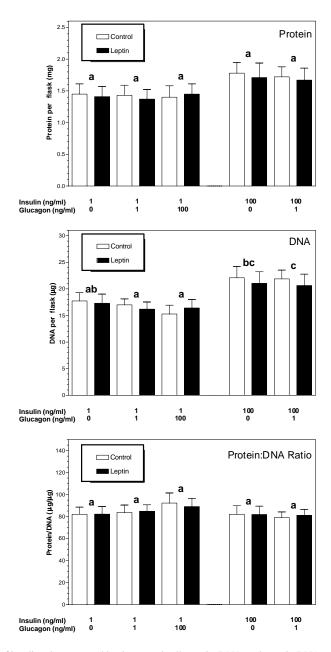


Fig. 3. Influence of insulin, glucagon, and leptin on total cell protein, DNA, and protein:DNA ratio in porcine hepatocytes. Following indicated incubations and removal of media, cell monolayers were washed with glucose-free medium and cells were scraped and frozen. Protein and DNA content were determined as described in Section 2. Data are means and S.E.M. for duplicate cultures from the five animals represented in Fig. 1. Means with different letters differ, P < 0.05.

when glucagon was elevated from 0 to 1 ng/ml. Therefore, high insulin levels blunted the inhibitory effect of glucagon on glycogen deposition. Addition of 200 ng/ml rh-leptin for 24 h to hepatocytes cultured over a range of glucagon (0, 1, and 100 ng/ml) and insulin concentrations (1 and 100 ng/ml) did not alter glycogen content.

3.3. Protein and DNA

Under serum-free conditions, there was a tendency for increased cellular protein and DNA in pig hepatocyte cultures when insulin was added above the basal level (Fig. 3). When the overall effect of insulin was separated by ANOVA considering two levels of insulin across all levels of glucagon, there was a significant 21 and 29% increase in protein and DNA (P < 0.005), respectively, in high insulin cultures compared to low insulin cultures. In contrast, the protein:DNA ratio was relatively constant for all cultures, regardless of the statistical analysis used. Addition of 200 ng/ml rh-leptin for 24 h to hepatocytes cultured over a range of glucagon (0, 1, and 100 ng/ml) and insulin concentrations (1 or 100 ng/ml) did not alter cellular protein or DNA levels.

3.4. Dexamethasone effects

In two experiments, hepatocytes were maintained with 10^{-6} and 10^{-7} M dexamethasone to determine the influence of glucocorticoid level on glycogen deposition and KB production (Fig. 4). Compared to cells maintained with 10^{-7} M dexamethasone, KB production was elevated (32–95%) in all conditions when the dexamethasone was present at 10^{-6} M. In contrast, hepatocyte glycogen content was markedly reduced by the higher level of dexamethasone (viz. 10–68% decrease); this appears to be most apparent in the presence of high glucagon. Nevertheless, at high levels of insulin ($100 \, \text{ng/ml}$), the relative glycogen deposition in 10^{-6} M compared to 10^{-7} M dexamethasone, although decreased, was close to unity, indicating that when glycogen levels were maximally stimulated by insulin, dexamethasone inhibition was attenuated. Addition of rh-leptin for 24 h had no influence on the relationship of dexamethasone to insulin and glucagon with respect to glycogen deposition and ketogenesis.

4. Discussion

To our knowledge, these results represent the first direct demonstration of the control of KB production and glycogen storage by insulin, glucagon, and dexamethasone in primary cultures of hepatocytes from post-neonatal pigs. Additionally, the possible influences of leptin on porcine hepatocyte metabolism have not been previously investigated. In the present study, carnitine (1 mM) was added to all culture medium to ensure that fatty acid entry into mitochondria was not limited by carnitine availability [32]. However, in preliminary studies, we did not observe any consistent influences of carnitine addition on either KB production or glycogen deposition. The reported data are expressed on a per milligram cell protein basis, as high insulin levels (100 ng/ml) were associated with increased protein and DNA content. Similar increases in protein and DNA have also been reported in

Insulin (ng/ml)

Glucagon (ng/ml)

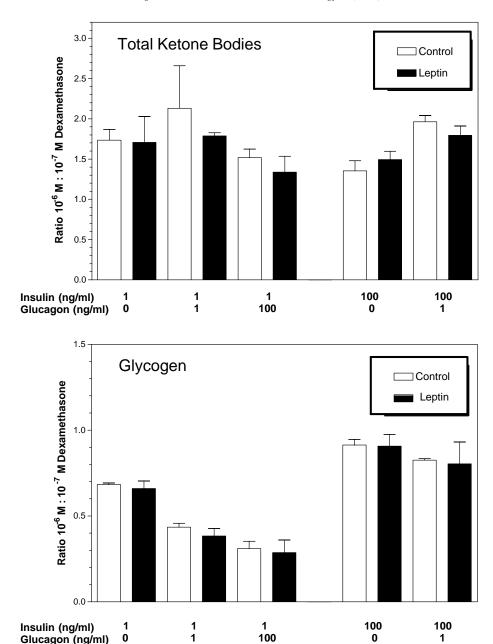


Fig. 4. Influence of dexamethasone, insulin, glucagon, and leptin on ketogenesis and glycogen deposition in porcine hepatocytes. Monolayers were maintained in either 10^{-6} or 10^{-7} M dexamethasone in the presence of different concentrations of insulin and glucagon for 72 h; rh-leptin was added during the final 24 h of incubation. Data are the means and S.E.M. of duplicate cultures for two separate experiments and represent the relative values for ketone production or cellular glycogen for cells maintained in $10^{-6}\,\mathrm{M}$ dexamethasone compared to those maintained in 10⁻⁷ M dexamethasone. No statistical analyses were performed.

1

0

100

1

insulin-supplemented cultured rat hepatocytes [33] and are likely to be reflective of enhanced cell attachment. Further, the initial cell density (160,000 cells/cm²) was relatively high and culture medium contained dexamethasone and DMSO with no other serum growth factors. While the regulation of hepatocyte proliferation is complex, and some cell division might be possible, these culture conditions would tend to attenuate DNA synthesis and cell proliferation and promote differentiated function in hepatocyte monolayer cultures [34–36].

Ketogenic capacity of liver from newborn pigs is known to be much lower than in other mammalian species [5,37,38] and low ketone production persists in the adult pig [7]. This has been attributed to a number of factors including low mitochondrial HMG-CoA synthase activity and sensitivity of CPT-1 to inhibition by malonyl Co-A [7,39,40]. Early in vivo studies with starved neonatal, miniature, or pancreatectomized pigs [41–44] suggested that mild ketosis encountered was modulated by insulin and glucagon.

Our results indicate that ketogenesis in hepatocytes isolated from 60 kg pigs is regulated by glucagon and insulin such that the higher the glucagon:insulin ratio, the higher the KB production. Ketone levels in the present study were similar to previously reported values for hepatocytes isolated from newborn and 15-day-old piglets [5,8], but markedly lower than ketogenesis in liver preparations from mature rats [7], ruminants [45,46], and humans [47]. The low rate of β-oxidation capacity has been ascribed to a propensity for fatty acid esterification versus oxidation in piglets [5]. Furthermore, low ketogenic potential in the piglet liver [5,7,48] compared to rat liver suggested that fatty acid carbon could flow via alternative pathways in pigs [8]. Although acetogenesis has been proposed as an alternative route to ketogenesis [9,10], serum acetate does not vary with metabolic state nor replaces ketogenesis in piglets infused with labeled hexanoate [49]. The influence of hormones on acetogenesis has not been investigated and acetate was not measured in the present study.

Glucagon enhancement of in vitro ketogenesis has been consistently reported in rat [50] and human [47] hepatocytes, but not in bovine hepatocytes [46]. In contrast, ketogenesis was only marginally increased in hepatocytes isolated from newborn pigs during short-term incubations with glucagon [5,51]. Here, we have demonstrated a marked enhancement of ketogenesis with low levels of glucagon, even in the presence of high insulin. In contrast, insulin antagonizes the ketogenic effects of glucagon in isolated [50] and cultured hepatocytes from fed rats [52], whereas insulin had marginal [32] or no effects [50] when present as the only hormone. Insulin was without effect on the rate of production of acid-soluble materials from palmitate in sheep [45] and bovine hepatocytes cultures [46]. We have found an inhibitory effect of insulin on ketogenesis in cultures of hepatocytes from pigs either in the absence or presence of glucagon (1 ng/ml). While the activity of hepatic HMG-CoA synthase, the primary regulator of ketone production, is low in pigs compared to other species [40], insulin would be expected to further down regulate HMG-CoA synthase expression levels [53].

The observed range in KBR is consistent with that observed in cultured rodent hepatocytes [54,55] and in blood levels of normal pigs [56,57]. Ketone body ratio, is an indicator of mitochondrial [NAD]:[NADH] which has been used both in vivo [56,57] and in vitro [54,55] to assess oxidation potential in normal and stressed states. Indeed, low in vivo KBR levels (<0.6) are indicative of increased risk of pathology and mortality in compromised humans and pigs [56,58]. In the present study, high levels of insulin were associated with a marked reduction in KBR, as well as a reduction in total ketone production. A relative

increase in NADH would result in an inhibition of enzymes that would promote TCA cycle activity, such as citrate synthase, α -ketoglutarate dehydrogenase and pyruvate dehydrogenase [59]. Thus, with high insulin, a shift towards glycogen deposition with a concomitant reduction in ketogenesis is observed in porcine hepatocytes. In contrast, Agius et al. [32] reported no change in KBR with insulin addition, while more recently it has been suggested that in rat hepatocytes, glucagon addition was associated with an increase in KBR [54]. Thus, the net effect of altering the insulin:glucagon ratio would be similar for rat and pig hepatocytes.

Changes in porcine liver glycogen parallel the insulin:glucagon ratio in vivo [43]. For example, mildly hypothermic newborn pigs, with higher levels of glucagon, had lower concentrations of liver glycogen [60]. To our knowledge, no direct determination of insulin and glucagon regulation of glycogen content in pig hepatocytes is available. In rat hepatocytes, insulin has long been known to increase glycogen deposition [11–16,61] while glucagon activates glycogenolysis [61,62] and inactivates glycogen synthase [63]. Further, insulin and glucagon have been shown to exert antagonistic effects on glycogen synthesis in rat hepatocytes [64]. We have demonstrated that in the absence of extrinsic factors, insulin alone or in the presence of glucagon increased glycogen content of primary culture of pig hepatocytes while glucagon acutely decreased it in a dose-dependent manner. Moreover, by altering the insulin:glucagon ratio the levels of glycogen could be varied over a 20-fold range.

Studies from several laboratories have shown that glucocorticoids also play a role in the regulation of ketogenesis in rat hepatocytes while less is known about the regulation of glycogen deposition by dexamethasone. In hepatocytes from adult rats, dexamethasone $(1-100\,\mathrm{nM})$ increased the rate of formation of ketone bodies in the absence of insulin; this short-term effect $(2\,\mathrm{h})$ was independent of the presence of dibutyryl cyclic AMP [32], although in the presence of insulin, dexamethasone was inhibitory. In that study, V_{max} of CPT-I and its affinity for palmitoyl-CoA were not affected by dexamethasone [32], suggesting that the control exerted by dexamethasone on ketogenesis is via mitochondrial HMG-CoA synthase. Indeed, dexamethasone has been shown to increase liver HMG-CoA synthase mRNA levels in fasted rats [53].

Synergistic stimulation by insulin and dexamethasone on the rate of glycogen synthesis and cellular glycogen content in hepatocyte monolayers has been reported previously for rat hepatocytes [32,65]. However, in the present study, glycogen content in pig hepatocyte monolayers was considerably decreased by long-term culture with higher dexamethasone levels $(10^{-6} \, \text{M} \, \text{versus} \, 10^{-7} \, \text{M})$ when the insulin level was low, although the effect was less evident with a high insulin level $(100 \, \text{ng/ml})$. Likewise, in the absence of insulin Baqué et al. [66] demonstrated net glycolysis as a function of increased dexamethasone concentration primarily due to activation of phosphorylase activity in rat hepatocytes. Thus, the effects of dexamethasone on ketogenesis and glycogen content were similar to the effects of glucagon, suggesting that high levels of dexamethasone may enhance cyclic-AMP production. In summary, dexamethasone appeared to decrease insulin sensitivity with regard to ketogenesis and glycogen content in primary culture of pig hepatocytes.

Leptin appears to exert several direct effects on peripheral tissues through leptin receptor signaling [67]. The potential role for leptin in modifying ketogenesis and glycogen deposition in liver has not been assessed in swine, although leptin is produced and secreted by

porcine adipocytes [68]. In our hands, leptin did not have a long-term (24 h) direct physiological effect on ketogenesis, redox state, or glycogen deposition in primary culture of pig hepatocytes, throughout a wide range of insulin and glucagon concentrations. In addition, we have not observed short-term effects (<6 h) on glycogen synthesis or degradation (T.J. Caperna and A.E. Shannon, unpublished observation). The use of rh-leptin should not invalidate the lack of response in the parameters evaluated, since swine leptin amino acid sequence bears high homology (85%) with human leptin [68]. Preliminary studies with recombinant porcine leptin (provided by T.G. Ramsay) also indicated a lack of effect in porcine hepatocytes. Furthermore, human recombinant leptin promoted angiogenesis in porcine aortic endothelial cells [69] and altered gene expression and metabolism in porcine pituitary cells [70]. The lack of effect of leptin on hepatocytes in vitro suggests that metabolic effects of leptin on liver tissue may be indirect via the CNS and/or feed intake in swine. The suppressive effects of leptin on phosphorylase activity and glycogenolysis and synergism with insulin have been previously reported for rat hepatocytes cultures [16]. Ceddia et al. [71] also demonstrated a reduction in glucagon induced glycogenolysis, while Nemecz et al. [72] demonstrated both insulin and glucagon-like effects of leptin on hepatic energy metabolism. Given the magnitude of the responses (in glycogenesis and ketogenesis) observed by varying levels of insulin and glucagon, any antagonistic or synergistic effects of leptin with either of these polypeptide hormones should have been observed in the present experiments, if any interaction exists between leptin and insulin or glucagon.

Direct effects of leptin on hepatocytes would presumably be mediated via the long form of the leptin receptor. Leptin receptor mRNA is present in swine liver [73], and we also have evidence (by RT-PCR) that both long and short forms of the leptin receptor are present in these cultured hepatocytes (M.P. Richards and T.J. Caperna, unpublished observation). Although the long form of the leptin receptor is expressed, the concentration of receptors may be too low for leptin to have any direct physiological effects on swine hepatocyte metabolism. Further study will be required to determine if the hepatic leptin receptor is indeed functional in pigs and whether leptin plays a role in swine hepatic energy metabolism as demonstrated for other species.

Acknowledgements

The authors wish to thank D. Parsons and J. Piatt for assistance with the handling of animals. This research was supported by fellowships from NATO Science Program and Ministry of Education and Culture of Spain (I.F.-F).

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